Effect of Cadaverine on *Lycopersicon esculentum* under multiple stress conditions.

SHORT SYNOPSIS

Submitted in partial fulfillment of the requirement of the degree of

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by

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15/Ph.D/020

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Certificate

I hereby declare that the short synopsis "Effect of Cadaverine on *Lycopersicon esculentum* under multiple stress conditions" being submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Department of Biotechnology under faculty of Engineering and Technology of Manav Rachna International Institute of Research & Studies, Faridabad during the academic year 2015-16.

It is a bona fide record of original work carried out under the supervision of Dr. Pushpa C. Tomar and has not been submitted in full or part to any other university or institute for degree or diploma. Further, it is certified that short synopsis is plagiarism free and proof has been submitted.

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Recommendation

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ABSTRACT

Abiotic stresses are a major threat for agricultural productivity. Several strategies have been developed to improve such stresses like crop salt tolerance and heavy metal stresses, and in regard to this the effects of polyamines (PA) have been well established. Cadaverine (Cad), a structurally different diamine as that of putrescine and having an independent biosynthetic pathway with respect to other polyamines is regarded as a growth regulator and follows the same mode of action. Tomato (*Lycopersicon esculentum)* being one of the most significant food crop of India is required to sustain such stresses. Several studies are established exhibiting the amelioration of stresses in different crops like brassica, burmund grass and leguminous plants by Cad. So this study will be carried out in order to explore its effect on tomato. Physiological analysis will be performed to observe the effect of Cad on biomass accumulation and increment of bio molecules. To assess the functions in multiple stress responses, the effects of exogenous Cad will be analyzed in tomato plants using 2D- electrophoresis. Signature proteins involved in biological and biochemical processes will be identified. During cell rescue and defense mechanism, protein expressions involved will be studied. Taken together, these experiments will deliver a fine understanding of Cad-induced multiple stress resistance by proteomic methods providing a practical perception for improvising multiple stress tolerance in the global epoch.

Key words: Cadaverine, Lysine, PA, Proteomics, Stress, Tomato (*Lycopersicon esculentum)*

CONTENTS

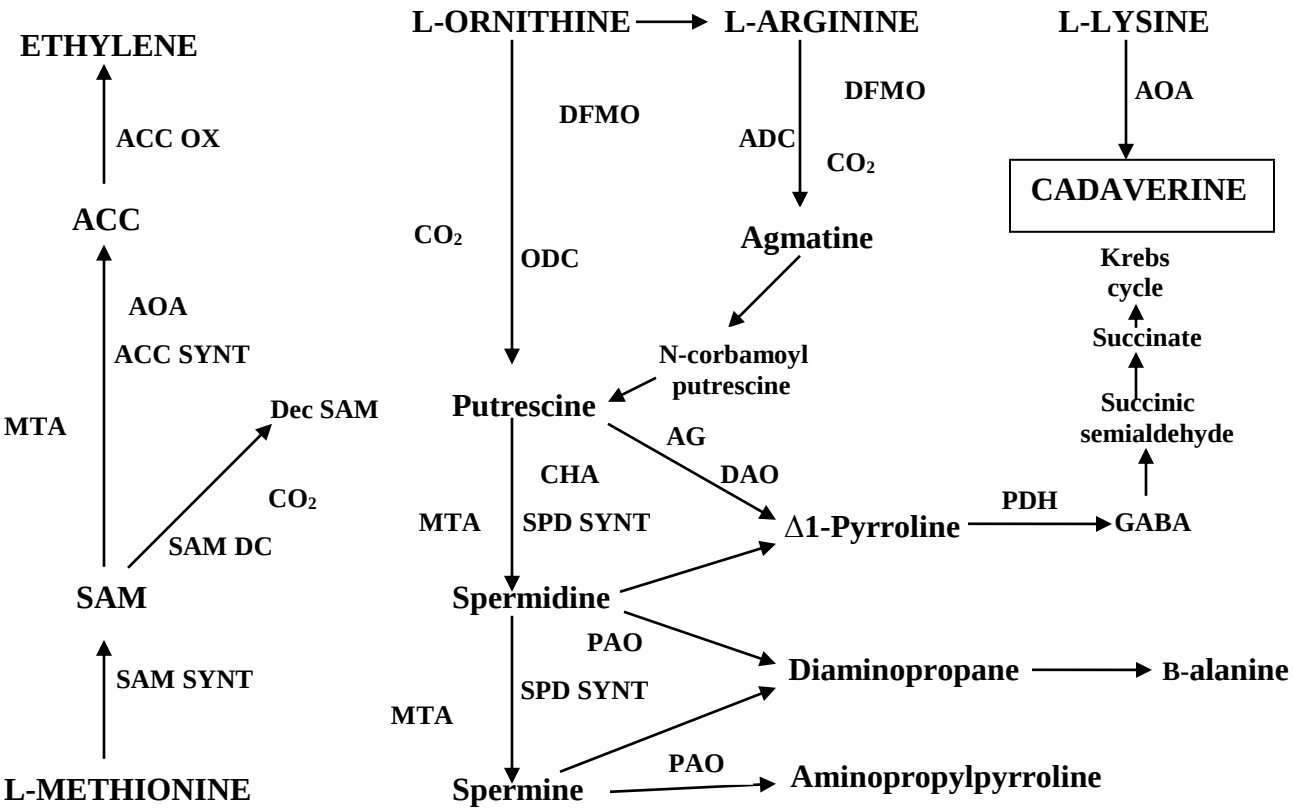
S. No.	Description	Page No.
1	Introduction	1-4
2	Literature Review	5-7
3	Description of Broad Area	8
4	Motivation of the Study	9
5	Objectives of the Study	10
6	Methodology to be adopted	11-12
7	Expected Outcome of the Research	13
8	Proposed Time Frame (Gantt Chart)	14-15
9	References	16-22

1. Introduction

Polyamines (PA) are small aliphatic polycations, ubiquitously present in all organisms. Functionally, these molecules exhibit their role in wide range of biological processes. Since last few years, molecular genetic studies have contributed immensely in understanding the transformed activity of enzymes in the synthesis of PA and their probable biological functions in plants. Previous studies have focused mostly on 3 biogenic amines: putrescine (Put), spermidine (Spd) and spermine (Spm), and their derivatives. The application of PA applied effects the patterns of senescence and morphogenesis providing a restrictive role of polyamines in these processes (Galston & Sawhney 1990). However, individual functions of putrescine, spermidine and spermine are uncertain due to their mutual conversion (Mattoo et al. 2010). Cadaverine (Cad), a structurally different diamine which has an independent biosynthetic pathway for its synthesis also comes under the family of PA. It is known as a growth regulator with similar mode of action. Cad also tends to accumulate in higher plants under several biotic and abiotic stresses. Over-expression of genes indulged in polyamine biosynthesis results in building up of PA and therefore, more stress tolerance. Cad is synthesized from the amino acid arginine (Figure 1). Agmatine is formed from ariginine and this reaction is catalysed by arginine decarboxylase (ADC), followed by its transformation into N-carbamoyl putrescine which ultimately gets converted into Cad using agmatine imino hydroxylase (AIH). Alternatively, Cad is also synthesized by a set of methionine synthesis through aspartate pathway. On the other hand, ornithine decarboxylase (ODC) uses lysine as a varied substrate for Cad synthesis.

Abiotic and biotic environmental stresses adversely affects crop yield (Yuan et al. 2016). One of the leading abiotic stresses is the salinity in the soil that owes to both natural reasons and an unsuitable agronomic operation. Tolerance to salt is a complex characteristic which is responsible for osmotic adjustments, exclusion of toxic ions, compartmentalization and morphological modifications (Munns & Tester 2008). A few recent studies have emphasized the effect of shoot tissue responses towards the excess salt concentration, by minimizing the accumulation of toxic ions in the leaf resulting in hampered growth and productivity of plant (Julkowska et al. 2014). Excess accumulation of salt ions present in the soil exert osmotic pressure which reduces the water uptake by the roots of the plants leading to excessive

accumulation of reactive oxygen species (ROS) (Jiang et al. 2016), and causes root membrane interruption (Gupta & Huang 2014).



Enzymes: ADC, arginine decarboxylase
ODC, ornithine decarboxylase
LDC, lysine decarboxylase
SPD SYNT, spermidine synthase
SPM SYNT, spermine synthase
DAO, diamine oxidase
PAO, polyamine oxidase
PDH, pyrroline dehydrogenase
SAM SYNT, SAM synthase
SAM DC, SAM decarboxylase
ACC SYNT, ACC synthase
ACC OX, ACC oxidase

Intermediates: SAM, S-adenosyl-methionine
DecSAM, decarboxylated SAM
ACC, 1-aminocyclopropane 1-carboxylic acid
GABA, γ-aminobutyric acid
MTA, 5-methylthioadenosine

Inhibitors: DFMA**,** difluoromethylarginine
DFMO, difluoromethylornithine
AG, aminoguanidine
AOA, aminooxyacetic acid
CHA, cyclohexylammonium sulfate

Fig. 1: Schematic outlines of the pathways of polyamine synthesis and catabolism.

Inflow of increased concentration of Na^+ to the root cells in general causes the outflow of cytosolic K^+ thus creating an imbalance of ion concentration leading to metabolic disorders (Witzel et al. 2009). Li et al. (2014) and Saha et al. (2015) stated that PA-PA interact with ion channels and contribute to ion homeostasis (Pottosin et al. 2014). Regulation of ROS homeostasis throughout salt stress is done by PAs by inducing the activation of antioxidant defense system.

Heavy metals accumulation has become a global concern due to crop irrigation by contaminated water by inhibiting nitrate reductase activity and decreasing organic nitrogen and protein contents in the leaf tissue (Hussain et al. 2015). The increased nitrogen reductase activity induced by PA is correlated with protein contents, plant growth and increased organic nitrogen (Singh et al. 2002). Ammar et al. (2008) reported that heavy metal concentration was higher in upper parts of plants like fruits as compared to the lower parts like leaves, roots etc. In contrast to this, there is a restriction in transportation of heavy metals to grain tissues in cereal crops and mostly remains in their roots. Cadmium taken up by plants ranges between 0.1-2.4 mg/kg (Alloways 1995). Higher concentrations can severely distress the growth of plant and biomass yield (Hussain et al. 2015). Gratão et al. (2008) when treated tomato plants with cadmium reported increased peroxidation of lipids, catalase activity, glutathione reductase activity and reduced activity of glutathion peroxidase enzyme (Ammar et al. 2007).

Interaction of PAs with plant proteins control plant adaptation (Yuan 2016). They stabilize cell structure and function by covalently binding with proteins in the presence of transglutaminase (Campos et al. 2013).

Proteomic analysis is one of the powerful tools to understand mechanism of plant stress tolerance (Skalák et al. 2016). Igrashi & Kashiwagi (2015) reported that stimulation of protein synthesis at the translational level could be due to PA because of the development of polyamine-RNA complex. It has been reported previously that when PA's get bound to charged spots on protein interfaces, they standardize the function of proteins through modulating protein-protein interactions electrostatically (Berwanger et al. 2010). Liu et al. (2015) stated that PA performs during stress tolerance mostly by modulating reactive oxygen species (ROS). They show a significant role in regulation inhibitor system or suppressing ROS

function. Thus, the exogenous application of Cad under stress is likely to exert some positive response in stressed plants which is not elucidated well.

The present study will be undertaken to provide some insight into Cad's regulatory mechanism on the growth of the tomato plant and its potential to mitigate the adverse responses induced under multiple stresses, generally realized by plants in field condition vis-à-vis to compare its response with NH_4NO_3. The proteomic analysis will be carried out on tomato plants that will reveal the increased multiple stress tolerance by Cad. In order to attain equilibrium of metabolic processes, plants respond to such environmental stresses by reprogramming their proteome and transcriptome which brings changes into the metabolism activity of the cell also (Sruthi et al. 2016). However, very less evidence is there to elucidate the precise mechanism through which PAs standardize environmental stress responses through a proteomic approach (Sang et al. 2017).

The tomato is one of the most significant vegetables and is sensitive to soil salinity and heavy metal stress. This study will investigate the expression of protein in tomato seedlings through 2D gel electrophoresis to comprehend the fundamental mechanism of stress tolerance imparted by cadaverine.

2. Literature Review

The study of polyamine response on growth and developmental aspect of plant is now being dealt by number of scientists. Several groups have worked upon different aspects of PA. Put, Spd and Spm are the three most verdant polyamines which show abundantly enhanced measure of abiotic stress (Yang et al. 2007). It has been observed in the majority cases that just one variety of the 3 polyamines show an apparent enhancement. For an instance, when Liu et al. (2006) treated callus of apple with salt, Put. levels amplify, whereas other PA like Spd and Spm experience insignificant changes. In a different study, it was conveyed that 18 different varieties of rice unveiled prominent deviations in Spm levels when they experience drought stress for a long period (Do et al. 2014). Such reports suggest that accumulation of polyamines is affected by variety of aspects like species of plants, their stress tolerance capacity, stress varieties and other environmental surroundings, and therefore the physiology of the inspected tissue/organs. This further specifies that the reason for getting differing and contradictory results is the presence of extensive PA dynamics under abiotic stress. The extent of PA pool may be related to the stress tolerance capacity, more emphasizing the importance of PA's in providing defense against stresses (Hatmi et al. 2015). Compelling proof specifies the involvement of PA in abiotic and biotic stress responses in plants. Certainly, genetic engineering of polyamine levels in plants has enhanced biotic and abiotic stress resistance in exemplary plants and crops effectively. There is a tendency to anticipate numerous existing challenges in agronomy to deal with environment modifications and uphold biological procedures and quality of fruits and vegetables that will be advanced by considering the polyamine pathway. As demonstrated by Tomar et al. (2013), lysine decarboxylase catalyzes lysine catabolism, which is developmentally regulated. Activation of multiple pathways due to binding of PA has resulted in increased salinity and draught tolerance in Burmund grass (*Cynodon dactylon)* (Shi et al. 2013). The Cad diverse presence in plant parts/products recommends it as a prospective candidate for taxonomic marker as well as for commercial manipulation along with plant growth and development.

Compared with other PAs, studies on role of Cad have been fragmented. Shalaby et al. (2000) discovered that concentration of Cad increased during germination in broad bean, chick pea and lupine. Garcia-Garcia et al. (2000) observed presence of small quantities of cadaverine in

table olives after three months of brining. Shen & Galston (1985) demonstrated Cad in corn coleoptiles, pea and tomato and in Datura (Carrizo et al. 2001). They have demonstrated the dependence of cadaverine titres on plant age and concluded that Cad declines progressively with age. Han et al. (2000) found elevated amount of Cad in immature seeds of citrus species. Piqueras et al. (2002) found high level of Cad conjugation under hyperhydric plants of carnation.

Fecker and his group (1993) observed the enhanced production of cadaverine in hairy root cultures of *Nicotiana tobacum*. Carrizo et al. (2001) found the occurrence of cadaverine in hairy roots of *Brugmansia candida*. Cadaverine application may induce rooting in the presence of ectomycorrhizal fungi in scot pine (Niemi et al. 2002). It may affect nodule metabolism by changing bacteriods (Vassileva & Ignatov 2002). Under heat shock, cadaverine level increased many fold compared with others in ice plants (Kuznetsov et al. 2002).

Recently, Goyal et al. (2016) found that pathogenesis-related protein 1b1 (PR1b1) is a chief tomato fruit protein receptive to chilling temperature and up regulated in high polyamine transgenic genotypes. Nambeesan et al. (2012) suggested that PA-mediated vulnerability to *Botrytis cinerea* in Tomato is interrelated to intrusion with the functions of ethylene in its defence. The ripening-related metabolic variations are both ethylene dependent and independent, and the fruit metabolome is under the control of multiple regulators, comprising ethylene and PA.

Cheng et al. (2009) found that accumulation of PA in transgenic tomato improves the tolerance to high temperature stress. Hence it postulates that PA shows a significant part in plant response to abiotic stress. Hichri et al. (2014) demonstrated that the zinc finger superfamily comprises of transcription factors that standardize multiple characteristics of plant development and shown to standardize abiotic stress tolerance recently. As described by Cheng et al. (2012); Spermidine distresses the transcriptome responses to high temperature stress in ripening tomato fruit. Many studies suggested that, transgenic gene lines or exogenous application showed a positive role of PAs in environmental stress tolerance (Kamiab et al. 2014). Comparative proteomic analysis could help in considering the mechanism of environmental stresses tolerance in the presence of PAs (Witzel et al. 2009). Jianming et al. (2015) reported enhanced tolerance to salinity-alkalinity stress in presence of exogenous spermidine by amending chloroplast antioxidant system and chlorophyll metabolism. By

eradicating reactive nitrogen and oxygen species in citrus leaves, which are bare to salinity stress proteins, tyrosine nitration and carbonylation can become effectively low (Tanou et al. 2014). Voltage dependent K^+ channels regulate the inhibition of the opening and closure of stomata (Liu et al. 2000) or H_2O_2 signals (Konstantinos et al. 2010) through PAs which results in transpiration restriction and reduction in water loss (Yuan 2016). As stated by Crespo et al. (2015) that NH^{4+} nutrition provides slight toxicity by increasing H_2O_2 accumulation, which functions as a signal, stimulating systemic attained acclimatization. Moreover, this study discovered the cross-talk between abiotic stress acclimatization (NH^{4+} nutrition) and resistance to consequent Pst infection.

3. Description of Topic

Prevailing environmental conditions are increasingly responsible for great losses in crop production every year. The problem of salinity is increasing day by day due to erratic irrigation and irrational fertilization. This is further aggravated due to metal pollution, especially Cd or Pb due to human activities. In fact, multiple stresses exerted on plants, are causing huge damage to crop production and human health. Abiotic stresses hamper plant growth by inhibiting germination, dry mass accumulation and change in biomass partitioning due to perturbed physiochemical processes result concomitantly into decline in growth and related metabolites. Most significant protective food crop of India is tomato. It is cultivated in 0.458 Million hectare (M ha) area with 7.277 Million metric ton (M mt) production and 15.9 metric ton/hectare (mt/ha) productivity. Though efforts are progressively made to produce stress resistant transgenic plants to sustain stress condition still this topic remains fascinating which are yet to be explored in plant sciences.

Polyamine like Cad is considered as growth regulator of plants and suggested to be anti-senescence and antistress agent (Galston & Sawhney 1990). These are aliphatic amines present widely in plants. Information about Cad physiological responses are scarce, except its involvement in variety of alkaloid synthesis. However, Cad a diamine structurally different from putrescine has been not evaluated for its potential for amelioration of stress effect in plant. Moreover, the Cad has independent biosynthetic pathway having lysine as precursor. A detailed study on growth and development of plant with different level of stresses; such as shock, to find response mechanism, to understand twin stress tolerance ability of the plants is required. This may provide insight for strategies and precise characteristic development of crop for sustainable growth and mitigating stress induced yield problem. Growth parameters will be screened under salinity and heavy metals stress for *Lycopersicon esculentum* widely grown in the region being hampered due to stresses like heavy metals, salinity, etc. This study will explore the expression of proteins in tomato plants differentially through Gel Electrophoresis to comprehend the fundamental mechanisms of Cad application in multiple stress resistance.

4. Motivation of the Study

This study is about identifying new potentials in agricultural biotechnology considering the current scenario of soil infertility in several states of India. This is extremely serious because India supports 18% of world's human population and 15% of the world's livestock population but has only 2.5% of world's land area. Biotic and abiotic stresses are the main cause of soil degradation. Salinity and heavy metal pollution is a great threat to soil fertility. Heavy metals enter to the soil-plant environment through anthropogenic sources. These substances adversely affect the productivity of soil, plants, animals and the entire environment if exceeds the certain limits. Among all the heavy metals, cadmium is highly toxic for both plants and animals. Compounds of cadmium are more soluble than other heavy metals rendering it more available for plant absorption where these could accumulate in edible plant part while lead is mostly present in top layer of soil due to deposition from air containing smoke from vehicles.

Similarly certain processes like weathering and deposition of oceanic salt carried in wind and rain are the major natural factors responsible for soil salinity. While human activities like land clearing and irrigation are other factors for salinization that change the hydrologic balance of the soil. This results in accumulation of the dissolved salts in soil water to an extent that inhibits plant growth.

Tomato being one of the most significant food crop of India is required to sustain stress condition is still a topic that remains fascination and yet to be explored in plant sciences.

Polyamines like Cadaverine (Cad) are aliphatic polycations that tend to accumulate in higher plants under several biotic and abiotic stresses over-express the genes responsible for stress tolerance. Physiological analysis will be performed to observe the effect of Cad on biomass accumulation, increment of bio molecules (photosynthetic pigments, total soluble proteins, organic nitrogen) and nitrate reductase activity which is well correlated with growth stress on tomato (*Lycopersicon esculentum)* plants will be determined and it will provide an insight by comparative proteomic and genomic studies comprehensively along with a better understanding about mechanism of Cad that will improvise the tolerance of multiple stress in tomato.

5. Objectives

As per the literature survey, the study is proposed with following aims and objectives:

1. To study cadaverine induced growth response of tomato plant under salinity and metal stresses.
2. To examine nitrate reductase activity in vivo in root and in leaf tissues and its regulation through cadaverine vis-a-vis in presence of NO_3^-/NH_4^+ to compare the response.
3. To determine endogenous concentration of Na^+/K^+, Cd or Pb and cadaverine correlating with physiochemical parameters.
4. To perfoem protein profiling through SDS-PAGE and N-terminal sequencing followed by measurement of endogenous PA including cadaverine in tissue, to demonstrate correlation with responses.
5. To reveal Cad responses in mitigating the multiple stresses, proteomic, genomic and physiological analysis will be carried out.
6. Lastly, examination of the quality and quantity of tomatoes in Cad treated plants under stress; if possible in the green house conditions will be done.

6. Methodology

- **Plant Materials and Treatments:**

Seeds of tomato (*Lycopersicon esculentum* var. Pusa Ruby, Pusa Rohini and Pusa Sadabahar) will be surface sterilized with 0.1% mercuric chloride ($HgCl_2$) and then sown. The seedlings will be grown under controlled conditions cultured with modified MS media (pH 6.4) containing various regimes of salinity and metal stress. The treatment of cadaverine along with salinity (NaCl) and heavy metals (Cd, Pb) will be given to seedlings since the first day of sowing, so as to find out any effect on nitrogen assimilation of plant under multiple stresses.

- **Measurement of Biomass, Chlorophyll content, NR and Organic Nitrogen:**

The germination of seeds will be counted after 24 and 48 hours of sowing. Random sampling will be done on 7^{th} day for various morphological and physiological measurements. Dry biomass will be estimated at 68℃ after drying the seedlings for 72 hours. Estimation of Chlorophyll content, Carotenoids, NR activity and Organic Nitrogen will be done. Na^+ K^+ ions will be estimated by Digital Flame Photometer and Cd and Pb absorption will be checked by Atomic spectrophotometer. The data will be mean of replicates along with ± SD. Further, student's'‘t’ test will be used to explore the implication of treatment.

- **Protein Extraction:**

Protein extraction will be performed through centrifugation and the supernatants will be precipitated with ice-cold acetone overnight. Further the resulting protein containing suspension will again be centrifuged followed by washing thrice with ice-cold acetone. At last, the protein pellets will be air-dried at room temperature and will be dissolved in rehydration buffer and then subjected to electrophoresis.

- **2D- Gel Electrophoresis:**

The protein profiling through SDS-PAGE and N-terminal sequencing followed by measurement of endogenous PA including Cad. in tissue to establish relationship with responses.

- **Image and Data Analysis:**

The 2-D gels will be scanned for spot detection. The intensity of each spot will be quantified on 2-D gels based on the volumes percentage (vol. %). Spots with major deviations will be considered differentially expressed.

- **Protein Identification:**

The identification of proteins will be done by MALDI-TOF.

- **Genomic Analysis:**

The genomic analysis will be done by real time PCR.

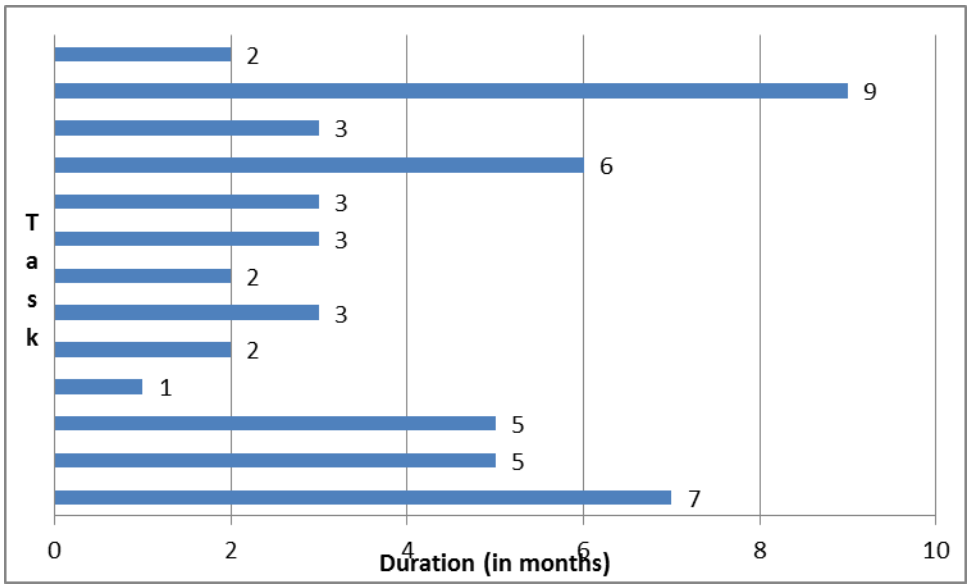
7. Expected Outcomes of the research

The study elucidates the precise role of cadaverine as growth regulator and/or inducer of nitrogen assimilation, hence affect plant growth and productivity under stress condition. Management of environmental stresses is a real challenge for plant scientists in agriculture. The other naturally occurring diamine Cad included in PA group of compounds, having an independent biosynthetic pathway is also considered to be plant growth regulator. Literature survey indicates cadaverine is either least studied or there is no precise information available about plant growth modulation under multiple stresses. This has not been evaluated extensively as compared to that of putrescine or spermine or spermidine in plant growth management especially under multiple stress conditions. Certain studies confirmed the assumption that the interaction between PA and ethylene may intermediate the effects of soil drying on grain filling of rice (Chen et al. 2013). PAs are nitrogenous molecules that show a firm role in most cellular processes during plant growth and development under physiological or biotic/abiotic stresses (Moschou 2012).

Though PA are implicated in anti-senescence response (Liu et al. 2000) yet cadaverine diamine is reported to be having no effect on senescence in barley (Srivastava 1987) or induce differential alkaloid synthesis (Carrizo et al. 2001) and increase membrane permeability of mitochondria. Since physiological relevance of polyamine increase under stress is least understood (Liu et al. 2000), Cad exogenous application under stress is likely to provide insight to understand the growth mechanism of plants, especially under twin stresses. Possibly the study will be providing understanding of mechanism of physiological responses of Cad, compared with putrescine. Ultimately, we will be demonstrating that how exogenous Cad application will effect tomato plant growth and attain multiple stress tolerance that could be allied with the under mentioned processes: (1) protein that is interrelated to photosynthesis and energy metabolism gets stimulated; (2) cell rescue and defense response mechanism gets activated by activating the antioxidant system to alleviate injuries caused during stresses; (3) stimulation of protein synthesis and degradation of damaged/misfolded proteins induced during multiple stresses. This study will provide an insight by comparative proteomics comprehensively and provide a better understanding about mechanism of Cad that will improvise the tolerance of multiple stresses in tomato plants.

8. Proposed Time Frame:

S.No.	Task	Start Date	Last Date	Duration (in months)
1	Course work	04-01-2016	04-08-2016	7
2	Literature Review	10-02-2017	31-08-2017	5
3	Gather Requirements	01-09-2017	28-02-2018	5
4	Short synopsis submission	01-03-2018	31-03-2018	1
5	Experiments installed	01-04-2018	01-06-2018	2
6	Growth study includes biomass measurement and metabolites estimation	02-06-2018	01-09-2018	3
7	Examination of NR activity	02-09-2018	01-11-2018	2
8	Detection of endogenous Na+/K+, Cd or Pb	02-11-2018	03-02-2019	3
9	Protein profiling	04-02-2019	01-05-2019	3
10	Proteomic and physiological analysis	02-05-2019	03-11-2019	6
11	Results interpretations	04-11-2019	02-02-2020	3
12	Examine the antistress property of cadaverine in the field condition	03-02-2020	01-11-2020	9
13	Long Synopsis submission	02-11-2020	31-12-2020	2



Gantt Chart as per the tentative dates.

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